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<td><strong>Author(s)</strong></td>
<td>Lee, PPW; Mao, H; Yang, W; Chan, KW; Ho, MHK; Lee, TL; Chan, JFW; Woo, PCY; Tu, W; Lau, YL</td>
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Penicillium marneffei infection and Impaired Interferon-gamma Immunity in humans with Autosomal Dominant Gain-of-phosphorylation STAT1 mutations

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Short title: Penicilliosis in children without HIV

Keywords: Penicillium marneffei; penicilliosis; chronic mucocutaneous candidiasis; STAT1; interferon-gamma; primary immunodeficiency

Word count: 1085
Capsule summary:

*Penicillium marneffei* is an AIDS-defining illness. We provide the first identification of autosomal dominant gain-of-phosphorylation *STAT1* mutations causing defective interferon-gamma and Th17 immunity in patients with penicilliosis, an invasive mycosis endemic in Southeast Asia.
To the Editor:

*Penicillium marneffei* (PM) is a pathogenic fungus endemic in Southeast Asia. PM was an extremely rare pathogen in human before the HIV epidemic, but following the exponential rise in HIV prevalence in Southeast Asia, penicilliosis emerged as a clinically significant opportunistic infection and is classified as an AIDS-defining illness. Less commonly, penicilliosis occurs in patients with other immunodeficiencies, such as severe combined immunodeficiency, common variable immunodeficiency, hyper-IgM syndrome, hyper-IgE syndrome, the presence of anti-IFN\(\gamma\) autoantibody, diabetes mellitus, immunosuppressive therapy, and solid organ or hematopoietic stem cell transplant. Affected individuals often have disseminated disease with rapid progression to multi-organ failure and death.

We previously reported 5 Chinese HIV-negative children and teenagers with disseminated penicilliosis. Four had co-existing chronic mucocutaneous candidiasis (CMC) since infancy, and one of them was genetically confirmed to have autosomal dominant hyper-IgE syndrome (AD-HIES). For the remaining 3 patients, a search for genetic defects in *CARD9, AIRE, STAT3, IL12B, IL12RB1, IFNGR1* was unrevealing. The co-existence of CMC and systemic penicilliosis suggested a possible functional defect of Th17 immune response in these patients. Recently, AD gain-of-function
missense mutations of STAT1 have been identified in several multiplex kindreds displaying CMC, autoimmunity and squamous cell carcinoma.\textsuperscript{4-9} We hypothesized STAT1 as a candidate gene, and we sought to determine the cellular response to STAT1 activation in these patients. Consent for genetic diagnosis and functional studies was obtained from parents, and the study was approved by The Institutional Review Board of The University of Hong Kong / Hospital Authority Hong Kong West Cluster.

P1, P2 and P3 were 3 unrelated Chinese children, and their clinical presentations and immunological profile were previously reported in detail.\textsuperscript{3} The core features and genetic findings of the patients and their parents are listed in Table 1 and Fig E1 (Online Repository). Heterozygous missense mutation in STAT1 was identified by Sanger sequencing in P1 (c.800C>T, p.A267V) and P3 (c.863C>T, p.T288I), and total exome sequencing in P2 (c.1074G>T, p.L358F; Online Repository). p.A267V is a known mutation while p.T288I and p.L358F are novel, but missense mutations involving the same amino acid residues (p.T288A and p.L358W) were reported in patients with CMC.\textsuperscript{4,6} Multiple sequence alignment of STAT1 orthologs (HomoloGene, NCBI) showed that all residues are highly conserved in animals except zebrafish for A267 and T288, and chicken for L358.
Missense mutations affecting the STAT1 coiled-coil domain identified in patients with AD-CMC have been demonstrated to be gain-of-function mutants with increased tyrosine-701 residue phosphorylation and enhanced $\gamma$-activated sequence (GAS) promoter binding activity. We compared the level of STAT1 phosphorylation in patients with healthy controls by flow cytometric analysis of intracellular phosphorylated STAT1 (pSTAT1). PBMC from patients and controls were stimulated with recombinant human IFN$\alpha$ (40,000 IU/ml) or IFN$\gamma$ (5,000 IU/ml) for 20 min. Compared with normal controls, lymphocytes from all patients demonstrated significantly higher percentage of pSTAT1+ cells and increased phosphorylation intensity in response to IFN$\alpha$ and IFN$\gamma$ stimulation (Fig 1A and B, Fig E2 in the Online Repository). The kinetics of STAT1 dephosphorylation was studied in P1. When treated with tyrosine kinase inhibitor, almost all STAT1 in control cells was dephosphorylated by 30 min; whereas about 50% and 25% of STAT1 in patient cells remained phosphorylated at 30 and 60 min respectively, indicating prolonged STAT1 phosphorylation in patient cells (Fig 1C). A missense mutant affecting residue L358 was previously shown to cause delayed dephosphorylation as well.

Next, we determined the proportion of IFN$\gamma$ and IL17A-expressing T-cells in PMBCs activated by overnight incubation with PMA (100 ng/ml) and ionomycin (1 $\mu$g/ml) in the
presence of Brefeldin A. Patients had significantly lower CD3+/IFN$\gamma$+ T-cells (14.8±1.5% vs 43.3±12.8%, p<0.01) and CD3+/IL17A+ T-cells (0.30±0.11% vs 2.15±1.41%, p=0.01; Fig. 1D) compared to normal controls. Finally, we evaluated the capacity of IFN$\gamma$ production towards fungal stimulation in P1 and P2. PBMCs were co-cultured with *Candida albicans* or PM for 2 days, and supernatants were collected for IFN$\gamma$ assay (FlowCytomix, Bender MedSystems). Compared with normal controls, P1 and P2 produced much lower IFN$\gamma$ towards both fungi (Fig. 1E). Production of other cytokines (IL1$\beta$, IL6, TNF$\alpha$ and MIP1$\alpha$) was studied in P1, and was comparable with normal controls. (Fig E3, Online Repository).

Previous studies demonstrated that patients with CMC caused by gain-of-phosphorylation *STAT1* mutations had impaired Th1 and Th17 response as a result of defective signaling through the IL12 and IL23 pathways. Majority of these gain-of-phosphorylation mutants are located in the coiled-coil domain and two in the DNA-binding domain. Impaired dephosphorylation of STAT1 enhances gamma-interferon activation factor (GAF)-dependent cellular response to IFN$\alpha$/IFN$\gamma$, and IL27, which are repressors of Th17 development from naïve T-cells. The enhanced response mediated by STAT1 probably impairs Th17 immunity.

The identification of *STAT1* and *STAT3* mutations in patients with systemic penicilliosis
suggests the importance of Th1 and Th17 immune response against PM. It is generally believed that PM establishes diseases in the lungs following inhalation of conidia, and disseminates in the form of intracellular yeast via the reticuloendothelial system. The activation of macrophages by IFN$\gamma$ is essential for their fungicidal activity against PM through the production of nitric oxide. While PM infection was self-limiting in wild-type mice, all IFN$\gamma$-knockout mice died of systemic mycosis. In humans, individuals with anti-IFN$\gamma$ autoantibody suffered from disseminated penicilliosis. Our experiments showed that lymphocytes of P1 and P2 exhibited defective IFN$\gamma$ production to PM in vitro. To our knowledge, this study shows for the first time that a primary defect in IFN$\gamma$ and IL17 immune response may be accountable for human PM infection. Penicilliosis should be regarded as an indicator of underlying primary immunodeficiency in HIV-negative individuals after excluding secondary causes.

It is worth noting that impaired IFN$\gamma$ and Th17 response in patients with gain-of-phosphorylation $STAT1$ mutations can predispose them to invasive mycosis as well as a range of bacterial and viral infections. Apart from penicilliosis, disseminated aspergillosis, candidemia, disseminated histoplasmosis and recalcitrant cutaneous fusariosis were reported. P2 and P3 had recurrent sinopulmonary infections caused by respiratory viruses and encapsulated bacteria, which was also similarly described by
Uzel et al\textsuperscript{6} and Takezaki et al.\textsuperscript{7} Of note, P3 had tuberculous lymphadenitis, recurrent herpes zoster and EBV-associated hemophagocytosis, supporting previous observation that AD gain-of-phosphorylation STAT1 mutations are associated with susceptibility to mycobacterial and herpes virus infections.\textsuperscript{8} Autoimmunity such as hypothyroidism, autoimmune hepatitis, systemic lupus erythematosus and type I diabetes mellitus, as well as malignancy such as esophageal carcinoma can lead to significant morbidities to this group of patients. The infectious disease susceptibility and phenotypic spectrum of AD-CMC caused by \textit{STAT1} mutations are wider than previously believed, revealing the divergent roles of STAT1 in host-pathogen interaction, immune tolerance and carcinogenesis.

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*These authors contributed equally to this work.

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REFERENCES


Table 1  Core clinical features of 3 patients with systemic *P. marneffei* infection and *STAT1* mutations.

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<tr>
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<td>CMC, <em>C. albicans</em> and <em>C. tropicalis</em> otitis externa, disseminated PM</td>
<td>CMC, disseminated PM, disseminated aspergillosis</td>
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<tr>
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<td>Nil documented</td>
<td><em>M. tuberculosis</em></td>
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<tr>
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<td>H1N1 influenza A respiratory infection with prolonged carriage, CMV pneumonitis</td>
<td>Recurrent herpes zoster reactivation, EBV-associated hemophagocytosis</td>
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<td>Nucleotide change</td>
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<td>c.1074G&gt;T</td>
<td>c.863C&gt;T</td>
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<td>Coiled-coil domain</td>
<td>DNA binding domain</td>
<td>Coiled-coil domain</td>
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<td>Carrier status of parents</td>
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<td>Not carrier</td>
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Figure 1. Gain-of-phosphorylation STAT1 mutations impaired IFN\(\gamma\) and IL17 responses.

**A,** PBMCs were stimulated with IFN\(\alpha\) or **B,** IFN\(\gamma\) and analyzed for intracellular pSTAT1 expression by gating on lymphocytes. The increase in %pSTAT1+ population in stimulated cells relative to unstimulated cells was calculated. Representative histograms are shown for P1 and a normal control.  

**C,** PBMCs from P1 were stimulated by IFN\(\gamma\) followed by treatment with staurosporine for 30 or 60 minutes. The percentage of intracellular pSTAT1 expression and mean fluorescence intensity (MFI) were determined in monocytes by flow cytometry.  

**D,** PBMCs were stimulated with PMA plus ionomycin and intracellular expression of IFN\(\gamma\) and IL17A in CD3+ T-cells was analyzed by flow cytometry. **E,** PBMCs were co-cultured with *C. albicans* (MOI of 5) or *P. marneffei* conidia (MOI of 1) for 48 hours, and IFN\(\gamma\) in the supernatant was quantified.